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Reversal of mutualism in a leafflower–leafflower moth association: The possible driving role of a third-party partner

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Short running title: Mutualism reversal in the leafflower moth

A major goal in the study of mutualism is to understand how co-operation is maintained when mutualism may potentially turn into parasitism. Although certain mechanisms facilitate the persistence of mutualism, parasitic species have repeatedly evolved from mutualistic ancestors. However, documented examples of mutualism reversals are still rare. Leafflowers (Phyllantheae; Phyllanthaceae) include ca. 500 species that engage in obligate mutualism with leafflower moths (*Epicephala*; Gracillariidae), which actively pollinate flowers, and whose larvae feed on the resulting seeds. We found that the Taiwanese population of the *Phyllanthus reticulatus* species complex was associated with six sympatric *Epicephala* species, of which three were derived parasites that induced gall formation on flowers/buds and produced no seeds. Notably, two parasitic species have retained mutualistic pollination behaviour, suggesting that the parasitism was likely not selected for to reduce the cost of mutualism. We suggest that the galling habit evolved as an adaptation to escape parasitism by a specialised braconid wasp. The tough gall produced by one species was virtually free of braconid parasitism, and the swollen gall induced by the other species probably prevents attack due to the larger airspace inside the gall. Our findings suggest that the presence of a third-party partner can greatly influence the evolutionary fate of mutualisms, regardless of whether the pairwise interaction continues to favour co-operation.

Additional keywords: *Epicephala*, gall, obligate pollination mutualism, parasitoid, *Phyllanthus reticulatus*, pollination behaviour.

INTRODUCTION

Mutualisms are widespread in nature and often play fundamental roles in diverse ecosystems. However, such relationships pose a problem for evolutionary theory, because they afford opportunities for overexploitation (Trivers, 1971; Axelrod & Hamilton, 1981; Bull & Rice 1991; Ferriere *et al*., 2002; Bronstein, Wilson & Morris, 2003; Sachs *et al*., 2004). Certain mutualisms feature host sanction or partner choice mechanisms that help stabilise co-operation by decreasing the fitness of non-cooperative individuals. For example, in pollination/seed consumption mutualisms such as fig–wasp and yucca–moth associations, plants selectively abscise flowers that contain high numbers of pollinator eggs relative to the amount of pollen deposited, thereby punishing individuals that impose heavy egg loads or those that do not pollinate (Pellmyr & Huth, 1994; Addicott & Bao, 1999; Goto *et al*., 2010; Jandér & Herre, 2010; Jandér, Herre & Simms, 2012).

Although mechanisms such as host sanctions may promote the stability of mutualisms, phylogenetic analyses of mutualist lineages often indicate that non-mutualistic taxa are nested within ancestrally mutualistic lineages (Pellmyr, Leebens-Mack & Huth, 1996; Hibbett, Gilbert & Donoghue, 2000; Bidartonto & Bruns, 2001; Lutzoni, Pagel & Reeb, 2001, Culley, Weller & Sakai, 2002; Als *et al*., 2004; but see Sachs *et al*., 2011, 2014 on the paucity of breakdowns in bacterial mutualisms), suggesting that mechanisms promoting stability over ecological time scales may be decoupled from those shaping macroevolutionary patterns. Mutualisms may breakdown either as the result of (1) mutualism reversal, whereby mutualists become parasites of the original mutualism, or (2) mutualism dissolution, whereby

mutualists evolve alternative life histories and live independently of the original partners. Early theoretical models emphasised the likelihood of the former process (Trivers, 1971; Axelrod & Hamilton, 1981; Bull & Rice, 1991), but examples of parasites that evolved from mutualists are rare. Such parasites include mycoheterotrophic plants that evolved from photosynthetic ancestors (Bidartonto & Bruns, 2001; Merckx & Bidartondo, 2008), and derived fig wasps and yucca moths that consume fig/yucca seeds without pollinating the plants (Pellmyr *et al*., 1996; Machado *et al*., 2001; Peng *et al*., 2008; Heraty *et al*., 2013). The rarity of mutualism reversal either suggests that parasite evolution is constrained in most mutualisms by mechanisms such as sanctions, or that parasites arise frequently but are evolutionarily short-lived.

Mutualism dissolution, by contrast, is much more widespread than reversals (Sachs & Simms, 2006). For example, plants have repeatedly abandoned partnerships with animal pollinators, shifting to wind- or self-pollination (Culley *et al*., 2002). Also, various lineages of mycorrhizal fungi have reverted to saprotrophic lifestyles and live independently of plant hosts (Hibbett *et al*., 2000). The shift to free-living status may be common because, over evolutionary time, the cost/benefit aspect of the mutualism becomes unfavourable as partners become difficult to encounter due, for example, to decoupled biogeographical history (Pellissier et al., 2012; Espíndola, Carstens & Alvarez, 2014), or the benefits gained from mutualistic partners become easily accessible in the environment, as exemplified by plants that abandon nutritional symbioses with mycorrhizal fungi or nitrogen-fixing bacteria in rich soils (Sachs & Simms, 2006).

Leafflowers, which are plants of the genus *Phyllanthus* sensu lato (including the embedded *Sauropus*, *Breynia* and *Glochidion*; Phyllanthaceae) include more than 1,200 species of monoecious trees and shrubs distributed worldwide, of which ca. 500 species engage in obligate pollination mutualisms with species-specific, seed-feeding *Epicephala* moths (leafflower moths; Gracillariidae; Kato, Takimura & Kawakita, 2003; Kawakita & Kato, 2009; Kawakita, 2010). Pollination by *Epicephala* is an active process. At night, female *Epicephala* moths collect pollen from male flowers using the proboscis, and subsequently deposit the pollen on the stigma of the female flower to ensure that the seed-feeding offspring of the moth will have a food source. The female proboscis of actively pollinating *Epicephala* species bears numerous hairs, absent from the proboscis of conspecific males, which likely facilitate pollen storage on the surface of the proboscis (Kawakita & Kato, 2006). After pollination, the female lays an egg in the flower that she has just pollinated, and the hatched larva consumes some of the resulting seeds, leaving the rest available for plant reproduction. The costs involved in such mutualism are, for plants, the attraction of pollinator adults to flowers (Okamoto *et al*., 2007; Svensson *et al*., 2008) and the seeds lost to pollinator larvae. For the pollinators, the costs are the time and energy spent in pollination and production of proboscis hair. Host sanction has been demonstrated in one species (*Glochidion acuminatum*); the plant selectively abscises flowers containing heavy egg loads (Goto *et al*., 2010).

In the present study, we report an example of mutualism reversal in the *Epicephala* moth lineage. Our preliminary observations on the *Phyllanthus reticulatus* Poir. species complex of Taiwan suggested the presence of multiple *Epicephala*

species with varying effects on the host. Based on field observations and molecular phylogenetic analysis, we first showed that six closely related *Epicephala* species occurred sympatrically on plants of the *P. reticulatus* species complex, of which three were mutualists and three were derived parasites. The latter did not provide benefits to the plants because they induced gall formation on flowers/buds without producing any seeds. We then studied the patterns of parasitism by specialised braconid wasps that attack moth larvae and suggested the possibility that the presence of a third-party partner (braconid wasps) may help explain the evolution of mutualism reversal in leafflower moths.

METHODS

FIELD OBSERVATIONS

Phyllanthus reticulatus is a shrub that is common along roadsides and forest edges in the tropical regions of Asia. Although long treated as a single species, the shrub has recently been split into two species, *P. reticulatus* and *P. microcarpus*, based on several distinct morphological and ecological characteristics (Luo *et al.*, 2011). Both species occur throughout our study sites in Taiwan, but in many of the populations that we studied, the plants showed intermediate characteristics suggestive of hybridisation, which rendered distinctions between the two species obscure. We thus considered that the plants belong (only) to the *Phyllanthus reticulatus* species complex; we used individual species names and mentioned putative hybrid status where appropriate.

Plants of the *P. reticulatus* species complex produce separate, small,

inconspicuous male and female flowers on leaf axils. Flowering and fruiting occurs throughout the year, and *Epicephala* moths emerge many times each year. The fleshy fruits contain 12–20 ovules, of which roughly half are consumed by a single pollinator larva. Although a previous study reported that pollination mutualism existed between these plants and *Epicephala* moths (Kawakita & Kato, 2009), close examination of plants suggested that many female flowers develop into galls that resemble fruits, but contain *Epicephala* moth larvae. Such galls can be classified into three distinct types: a small, tough gall with a dented surface (*E*. sp. C in Fig. 1); a swollen gall (with an internal airspace) that is distinctly larger than a normal fruit (*E*. sp. D in Fig. 1); and a gall with a weakly knobbed surface that is similar in size to a normal fruit (*E*. sp. E in Fig. 1). In all three types of gall, a single *Epicephala* larva develops by feeding on a single galled ovule; a gall may contain multiple *Epicephala* larvae if several moth eggs are laid in a single flower. The former two types of gall are abundant at our study sites, but the last type occurs at low density. We determined whether moth larvae occupying normal fruits, and galls of different types, represented distinct species. We thus reared adult moths from fruits and galls and delimited species using a molecular approach.

We also studied the behaviour of 37 adult *Epicephala* moths on flowers at night and recorded: (1) whether each moth displayed pollination behaviour prior to oviposition (a feature of pollinators in *Epicephala*); (2) the location of egg deposition; and (3) the number of ovipositions per visit to a single flower. Only individuals for which the entire behavioural sequence (from the approach to a flower, through to departure from the flower) was observed were included in analyses. The moths were

collected after observation, and were later microscopically checked in terms of proboscis pollen load. A hind leg of each specimen was stored in ethanol for use in subsequent molecular analyses. Moth observations were made in April and October 2008, March 2011, March and September 2012, April 2013, February and March 2014 and May 2015 over a total of ca. 50 hours, at seven locations in Taiwan (the location details are provided in Table S1).

MOLECULAR PHYLOGENETIC ANALYSIS

We sequenced upstream portions of the cytochrome oxidase subunit 1 (COI) gene of 113 reared adults of known larval biology and the abovementioned 37 field-collected females that exhibited recorded behaviours. We extracted genomic DNA from a hind leg of each specimen and performed polymerase chain reaction (PCR) and sequencing following the protocols of Kawakita *et al*. (2004) and Kawakita & Kato (2006). We used either the LCO and HCO primers (Folmer *et al*., 1994) or those described by Kawakita & Kato (2006) for PCR and sequencing. The obtained sequences have been deposited in DDBJ under accession numbers AB978376–AB978533 and LC059995–LC059998 and in BOLD under the project EPICE. The aligned matrix (780 bp) was subjected to maximum-likelihood (ML) phylogenetic analysis using Treefinder software (Jobb, 2008) and the substitution model chosen by the program. The robustness of the ML tree was validated by bootstrap analysis (1,000 replications) using the same program. We also conducted a Bayesian analysis using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) with substitution models chosen by MrModeltest 2.3 (Nylander, 2004). Trees were sampled every 100 generations, and the average

standard deviations of split frequencies calculated every 1,000 generations. Using the stoprule option, analyses were continued until the average standard deviations of split frequencies fell below 0.01, at which point the Bayesian chains were considered to have achieved convergence. Because the average standard deviations of split frequencies were calculated based on the last 75% of all samples, we discarded the initial 25% of sampled trees as burn-in. We confirmed that all of the analyses attained the stationary condition well before expiration of the burn-in period by plotting the ln-likelihood values of the sampled trees against generation time.

Because the above analysis suggested that several *Epicephala* species were present, we determined the phylogenetic relationships among species by sequencing the nuclear arginine kinase (ArgK) and elongation factor 1-alpha (EF1α) genes, in addition to COI, for 1 individual of each species, and compared the data with previously published sequences from 23 further *Epicephala* and related *Conopomorpha* species associated with a broad range of plants in the tribe Phyllantheae (Kawakita & Kato, 2009). We used previously developed ArgK and EF1α primers (Kawakita *et al*., 2004) and the laboratory protocols and phylogenetic analyses described above for COI. Analyses were performed both separately (on individual genes) and simultaneously (on the three genes combined) to check for possible conflicts among the data sets. Substitution models and rates were fitted separately for individual gene partitions upon ML and Bayesian analyses of the combined data. The accession numbers of sequences used in such analyses are given in Table S2.

INTENSITY OF PARASITISM BY A PARASITOID WASP

The field observations and molecular analyses described above indicated that three derived parasitic *Epicephala* species were responsible for production of the three gall types (one type per species). Because variation in gall traits, or in the galling habit in general, may be caused by selection imposed by natural enemies (Stone & Schönrogge, 2003; Bailey *et al*., 2008), we hypothesised that galling in *Epicephala* evolved to defend themselves from a specialist parasitoid, rather than as a response to a shift in the cost/benefit balance of the interaction of the moth with plants. To test this hypothesis, we focused on two *Epicephala* species: those that induce tough galls and those that induce swollen galls. These species were chosen because both gall types were abundant at our study sites, and because the defensive functions of these gall traits are relatively straightforward in both species.

The most prevalent natural enemies of *Epicephala* larvae are specialised *Bracon* wasps associated with most *Epicephala* species studied to date (Table S3). The wasps insert their ovipositors into fruits/galls containing later-instar *Epicephala* larvae and lay single eggs on the body surface of a moth larva (personal observation of S. Furukawa, Kyoto University). The wasp larvae develop by consuming the moth larvae externally, and emerge as adults from the fruits/galls. Gall toughness may render it impossible for the piercing wasp ovipositor to penetrate the gall wall, whereas the internal airspace of the swollen gall may increase the distance between the gall surface and *Epicephala* larvae inside galled ovules, rendering the wasp ovipositor too short for effective oviposition.

To test these possibilities, we first compared wasp oviposition intensities

and the actual parasitism levels between normal fruits and tough galls occurring on the same four *P. reticulatus* plants in the Hengchun population in March 2014 (no other gall type was evident in the Hengchun population). We searched for ovipositing wasps on the four plants during the daytime, for a total of 2.5 h, and whenever we encountered such wasps, we recorded whether ovipositions occurred into normal fruits or galls. The frequencies of ovipositions into fruits and galls were compared to the natural abundance levels of fruits and galls at the time of observation, calculated by randomly sampling branches bearing fruits and galls from the four plants (after observation), and later counting the fruits and galls. The fruits/galls were incubated at room temperature for > 3 days, dissected, and the parasitism rates were calculated by microscopically checking for wasp infestation of moth larvae.

Next, we sampled swollen galls from four putative hybrid individuals of the Longshui population in March 2014. If moth larvae inside larger galls are more likely to escape braconid attack, we expected that larger galls would have greater proportions of surviving larvae. However, gall size per se may be affected by parasitism, because induction of gall development by *Epicephala* larvae may cease at the point when the moth larvae die from wasp infestation. Thus, for each gall, we measured the size thereof (the horizontal width) and counted the numbers of infested ovules (as proxies for the numbers of moth larvae that initially infested each gall); *Epicephala* moth larvae; and braconid larvae/pupae. In some cases, moth larvae and/or wasp adults had already left the galls; however, the individual exit holes were distinguishable, based on surface structure (Fig. S1). Thus, we also counted the numbers of exit holes and included these in the tallies of moths/wasps inside galls.

For a subset of galls, we also measured the sizes of individual infested ovules to determine whether the increase in overall gall size was solely attributable to an increase in the volume of the internal airspace. We used these data to explore the effect of overall gall size on survival of *Epicephala* moth larvae. Statistical analyses were conducted using generalised linear mixed models (GLMMs), with individual plants serving as random factors. Analyses were conducted using the glmmML package of R version 3.1.0 (R Core Team, 2013).

Finally, to determine whether the braconid wasps reared from fruits and galls belong to the same species, we sequenced the upstream portion of the COI gene for a subset of the wasps obtained from swollen galls (n = 7), tough galls (n = 3) and normal fruits of *P. reticulatus* (n = 8) and *P. microcarpus* (n = 7). For comparison, we sequenced the COI gene for additional 24 *Bracon* wasps associated with 9 other *Epicephala* species spanning a broad phylogenetic range within *Epicephala* (see Table S3 for sampling details). DNA was extracted from legs of ethanol-preserved adult wasps. Sequences were obtained and phylogenetic trees constructed as described above for *Epicephala*. Obtained sequences have been deposited in DDBJ under accession numbers LC027116– LC027164.

EVOLUTION OF HAIRS ON THE PROBOSCIS

Because female proboscis hair development incurs a potential energetic cost, we determined whether any reduction in this trait was evident as mutualism shifted to parasitism. We counted the numbers of hairs on the proboscises of three to six female moths of each of the six *Epicephala* species found in the present study. We also

collected similar data for 16 of the remaining 22 *Epicephala* and *Conopomorpha* species included in the above phylogenetic analysis, for which specimens were available (1–3 moths per species; Table S4). For each moth, we removed the proboscis and divided it into the left and right galeae, which were then mounted in Euparal (Waldeck, Münster, Germany) on glass slides under cover slips, and the number of hairs per galea was microscopically counted. To correct for variation in body size, we also measured the head width of each moth to the nearest 50 μm. Sampling details and a summary of measurements are shown in Table S4.

RESULTS

Field observations and COI sequencing of the DNA of adults reared from fruits/galls indicated that six distinct species were associated with plants of the *P. reticulatus* species complex in Taiwan (Figs. 1, S2). *Epicephala* sp. A is a pollinator, which actively pollinates flowers and lays eggs in female flower pedicels (Fig. 1). Oviposition occurred once on each flower in all 11 moth individuals observed. Pollinated flowers develop into normal fruits, and a single larva consumes some of the developing seeds. *Epicephala* sp. B, the least common of the six species, is also a pollinator that produces normal fruits. It actively pollinates, and lays eggs superficially on the upper wall of the ovary (Fig. 1). This behaviour was repeated three times on a single flower, and three eggs per flower were thus laid per visit by each of the 2 moth individuals observed. *Epicephala* sp. C is a parasite that induces tough galls on female flower buds. None of the 10 moths observed exhibited any pollinating behaviour, and laid eggs in young female buds (Fig. 1), which eventually

developed into tough galls. No pollen was attached to the proboscises of ovipositing females (n = 10). *Epicephala* sp. D is also a parasite that induces swollen galls on female flowers. Notably, all the 5 female moths observed exhibited pollination behaviour similar to that of the pollinator species (Fig. 1). The eggs were superficially laid on the upper ovary wall of the female flower (n = 5). Abundant pollen was attached to the proboscises of all ovipositing females collected, indicating that they had collected pollen on male flowers. Pollination and oviposition occurred three times on each visit to a single flower by all the 5 moth individuals. Infested flowers developed into swollen galls (Fig. 3A,B) with internal airspaces (Figs. 1, 3C) within which ovules containing moth larvae developed into masses of endosperm-like tissue that lacked the features of normally produced seeds (e.g., a seed coat). Such irregular ovules were entirely consumed by the larvae of *E*. sp. D; thus, although we did not test whether the irregularly developed ovules retain the germination ability, the presence of *E*. sp. D was clearly not beneficial to the plant. Ovules that were not attacked by moth larvae inside the swollen gall remained undeveloped (Fig. 3C). *Epicephala* sp. E is (similarly) a galler that also displayed pollination behaviour (n = 5; Fig. 1). However, only two of the five ovipositing moths observed possessed pollen on the proboscis, suggesting that pollen collection may be occasionally omitted in this species. Oviposition occurred once per visit, and infested flowers developed into galls containing irregularly developed ovules typical of those induced by *E*. sp. D, but lacking the internal airspace. *Epicephala* sp. F is a pollinator that lays eggs into the apical stigmatic pits of female flowers (Fig. 1). Only one egg was laid per visit by all the 4 moth individuals observed. As with other *Epicephala* species studied to date

(Kato *et al*., 2003; Kawakita & Kato, 2009), moth behaviors of the six species are highly stereotyped, and thus, although the numbers of observations are limited for some species due to difficulty of encountering adult moths in the field, it is unlikely that other individuals display drastically different behavior from those documented above. Analysis of COI sequences identified six distinct clades (Fig. S2), which corresponded perfectly with the observed differences in adult behaviour and the characteristics of the fruits/galls from which adults were reared. Li & Yang (2015) recently described three *Epicephala* species associated with *P. microcarpus* in Hainan Island and mainland China, but the above six species do not match any of the species described by Li & Yang (2015) based on morphology of male and female genitalia.

Phylogenetic analysis of the 29 *Epicephala* and *Conopomorpha* species associated with a broad diversity of Phyllantheae plants indicated that the six species associated with the *P. reticulatus* species complex were monophyletic, and that the parasitic species were derived from pollinating ancestors (Fig. 2A). It is unclear whether mutualism reversal occurred only once, or many times, within the clade, because the level of statistical support at internal nodes was low (Fig. 2A). Reversion to parasitism also occurred in a clade of *Epicephala* moths associated with the weed *Phyllanthus*, as previously reported (Kawakita & Kato, 2009). The non-pollinating *Conopomorpha flueggella* associated with *Flueggea suffruticosa* (Kawakita & Kato, 2009; Hu *et al*., 2011) is embedded within *Epicephala* in our ML tree. However, it is not clear if this represents another case of mutualism reversal, because of low statistical support at the basal nodes (Fig. 2A). Female proboscis hairs (Fig. 2B) were absent in *Conopomorpha flueggella* and the *Epicephala* clade that is sister to this

species, but were present in the other mutualistic *Epicephala*. However, such hairs were absent in the three gall-forming *Epicephala* species found in the present study (Fig. 2C), indicating that the hairs were lost as the galling habit evolved.

Field observation of wasp oviposition on tough galls produced by *E*. sp. C, and subsequent dissection of the galls, indicated that the braconid wasp seldom attacked the larvae of *E*. sp. C (Table 1). In only one instance did we observe the braconid attempting to oviposit on the tough gall, but the wasp failed to pierce the gall wall. Rather, *E*. sp. C moths were regularly infested by a eulophid, *Aprostocetus* sp. (Fig. 3E), which was far less abundant on *E*. sp. A, that produces normal fruits. *Aprostocetus* wasps so far have not been found from *Epicephala* species associated with plants other than those of the *P. reticulatus* species complex. Braconid ovipositions into tough galls occurred significantly less frequently, and those by eulophids occurred significantly more frequently than expected by consideration of the natural abundances of fruits and galls (Fisher's exact test, $P < 0.001$ for both comparisons; Table 1). The same pattern was observed when actual parasitism levels (by braconids and eulophids) inside galls and fruits were evaluated (Fisher's exact test, $P < 0.001$ for both tests; Table 1).

In contrast, *E*. sp. D, which induces a swollen gall, was susceptible to braconid attack (Fig. 3D). However, this species was more likely to escape braconid parasitism when larger galls were infected. Overall gall size increased as more ovules were infested in each flower (GLMM, $n = 348$, $P < 0.001$; Fig. 3F), indicating that gall size was dependent on the number of moth larvae infesting the gall. In turn, the number of ovules infested per gall did not affect the size of infested ovules per se

(GLMM, n = 89, P = 0.180), indicating that the increase in overall gall size was not due to increase in the size of infested ovules but solely attributable to an increase in the volume of the internal airspace. In addition, galls containing parasitised moth larvae were smaller than those with intact moth larvae only (GLMM, n = 348, P < 0.001; Fig. 3F), suggesting that larval induction of gall development ceases as moth larvae become parasitised. We therefore consider that the number of infested ovules per gall is a better proxy of the gall size at which braconid ovipositions occur. Logistic regression of the parasitism rate (the proportion of moth larvae parasitised per gall) on the number of infested ovules (a proxy for gall size) showed that the parasitism level decreased as more larvae infested the gall, and hence, as galls became larger (GLMM, n = 380, P < 0.001; Fig. 3G).

Phylogenetic analysis of the *Bracon* COI sequences suggested that the wasps reared from fruits and galls of plants in the *P. reticulatus* species complex group into two separate clades (Fig. S3). There was a clear difference in the pattern of dorsal markings on the thorax and abdomen of male wasps between the two clades (Fig. S3), suggesting that each clade represents distinct species. The first species contained wasps reared from tough galls produced by *E*. sp. C (n = 3) and fruits of *P. microcarpus* produced by *E*. sp. F (n = 7). The second species contained wasps reared from fruits of *P. reticulatus* produced by *E*. sp. A (n = 8) and swollen galls produced by *E*. sp. C (n = 7) (Fig. S3). There was no support from the COI phylogeny that these two *Bracon* species are sister to each other (Fig. S3).

DISCUSSION

A major goal in the study of mutualism is to explain how co-operation is maintained, even when mutualisms may potentially turn into parasitism. Examples of parasites evolving from mutualistic ancestors offer opportunities to study the circumstances under which such evolution occurs, but documented examples of mutualism reversals are still limited (Pellmyr *et al*., 1996; Machado *et al*., 2001; Merckx & Bidartondo, 2008; Peng *et al*., 2008). The leafflower–leafflower moth association offers a new opportunity to study mechanisms underlying the shift to parasitism because parasitic *Epicephala* arose multiple times in the history of the mutualism (Fig. 2A). One such reversal involves the clade of *Epicephala* moths that shifted onto the weed *Phyllanthus* and lost pollination behaviour (Fig. 2A). Because the weed normally attains a 100% fruit set via pollination by abundant ants that forage for nectar (Kawakita & Kato, 2009), the benefit moths gain (seeds) are freely accessible without any need for the moths to pay the associated cost (pollination).

In turn, identifying the cause of the mutualism reversal found in the three galler *Epicephala* species of the present study is not straightforward. One possibility is that galling afforded a more cost-effective means of gaining resources than pollinating. However, the parasitic *E*. sp. D and sp. E both retain pollination behaviour besides investing in the chemical substances that induce the galls, indicating that the cost paid by gallers is not substantially lower, if it is lower at all, than that paid by the mutualistic ancestors. Alternatively, galling may improve the nutritional value of larval food, or allow the moths to circumvent selective flower abscission, and thereby increase larval survival. However, nutritional improvement or flower retention does not comprehensively explain some features of the galls, such as

toughness or presence of airspace inside the gall.

We hypothesise that the mutualism reversal evident in the present study is a by-product of an adaptation made by *Epicephala* moths to escape braconid parasitism. Proof that galling is an effective defence mechanism requires a comparison of mortality between gallers and non-galling ancestors at the time when galling evolved; we acknowledge that such analysis is impossible using long-established extant galler lineages. Nevertheless, the patterns of parasitism experienced by the galler species studied are consistent with the possibility that galling initially evolved as a defence, as often assumed in the case of galls produced by many other insects (Stone & Schönrogge, 2003; Bailey *et al*., 2008). For example, *E*. sp. C, bearing tough galls, is virtually free of braconid parasitism, whereas *E*. sp. D, with swollen galls, experiences higher survival when the galls have a larger internal airspace. Presently, neither species necessarily experiences lower parasitism than closely related mutualist species (overall parasitism rates are 41.7% (n = 240) and 27.7% (n = 1724) for the parasitic *E*. sp. C and D, respectively, and 22.5% (n = 151) for the mutualist *E*. sp. A, calculated based on the original data used for Table 1 and Fig. 3G), probably because enough time has elapsed for the parasitoid community to adapt to gallers. For example, *E*. sp. C is presently attacked by a eulophid wasp, and *E*. sp. D continues to suffer high-level braconid parasitism (Fig. 3G), possibly because the wasp ovipositor co-evolved (became longer) with increasing gall size. Escape from parasitoid attack by gall induction may be a common evolutionary trajectory in *Epicephala* because similar galls are produced by *Epicephala* moths associated with *Glochidion* in Japan and *Phyllanthus* in Madagascar, both distantly related to the *Epicephala* species

associated with plants of the *P. reticulatus* species complex (A. Kawakita and M. Kato, personal observations).

We do not know why *E*. sp. D and sp. E retain pollination behaviour despite development of a galling ability. One possibility is that although pollination is unnecessary, the behaviour cannot be easily lost because it is tightly integrated into the sequence of *Epicephala* oviposition. This was suggested by the observation that some individuals of *E*. sp. E did not have pollen on the proboscis; that they sometimes oviposited in buds; and that proboscis hairs were absent in both species. These findings indicate that selection towards pollen transport was relaxed. Unlike pollination behaviour, however, proboscis hair may be a labile character that can be quickly lost after galling evolves. In any case, the evolution of galling did not eliminate the cost of pollination behaviour. Whether proboscis hairs are associated with substantial costs (for example, impeding of feeding) remains to be determined. The proboscis constitutes less than 0.3% of moth body mass (A. Kawakita, unpublished data), and the microscopic structures evident on the surface thereof are unlikely to be costly, at least energetically (also see Pellmyr, 1997 for a discussion of the similarly small structural cost of the pollen-manipulating appendages of yucca moths).

Some theories of mutualism suggest that long-term persistence thereof is facilitated by mechanisms that stabilise the cost/benefit ratios of the interacting partners (Bronstein *et al*., 2003; Sachs *et al*., 2004). However, our findings lead to a hypothesis that factors extrinsic to the mutualism can have large effects on the evolutionary fate thereof, regardless of whether the pairwise interaction continues to

favour co-operation. In our present study system, derived parasitic *Epicephala* species were able to stably co-exist with related mutualistic *Epicephala* species, on a single host. However, if parasitic species limit the persistence of mutualist populations via processes such as resource competition or reproductive interference, it is possible that mutualism collapses solely via a process unrelated to the cost/benefit balance of the interaction. Our study thus highlights the need to explore how factors extrinsic to a mutualism may shape the macroevolutionary dynamics of that mutualism; such work will improve our understanding of mutualism stability.

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FIGURE LEGENDS

Figure 1. The six *Epicephala* moth species co-occurring on plants of the *Phyllanthus reticulatus* species complex in Taiwan. Plus (+) and minus (-) symbols indicate presence and absence, respectively. Some ovipositing females of *Epicephala* sp. E do not carry pollen on the proboscis (hence "±" under "Pollen on proboscis"). *Epicephala* sp. A, B, and F produce normal fruits containing viable seeds (drawn as filled ovules under "Flower fate"), whereas *E*. sp. C, D, and E induce galls that contain non-viable galled ovules (drawn as open ovules). Shaded areas inside galls indicate internal airspace. Fruits and galls are drawn proportional to their actual sizes. A putative hybrid between *P*. *reticulatus* and *P*. *microcarpus* is indicated as *P*. *ret*. × *P*. *mic* under "Host species".

Figure 2. Evolution of pollination behaviour in *Epicephala*. (A) Maximum-likelihood phylogeny of the genus *Epicephala* based on combined data from the COI, ArgK, and EF1α genes. Numbers at the branches are the maximum-likelihood bootstrap values followed by the Bayesian posterior probabilities. Nodes unsupported upon individual gene analysis are indicated as hatched lines, with support values in italics. The monophyletic clade containing the six species associated with plants of the *Phyllanthus reticulatus* species complex is highlighted in the shaded box. Mutualistic and parasitic lineages are coloured green and blue, respectively. Boxes located to the left of the terminal taxon names indicate the presence/absence of pollination behaviour (left; present when green) and hairs on the proboscis (right; present when green; open boxes indicate missing data). Major evolutionary events are indicated in

boxed notes. Genus abbreviations are: *E*., *Epicephala*; *F*., *Flueggea*; *P*., *Phyllanthus*; *B*., *Breynia*; and *G*., *Glochidion*. (B, C) Scanning electron micrographs of female proboscises of *Epicephala* sp. A (pollinator; B) and *E*. sp. D (galler; C).

Figure 3. Galls produced by *Epicephala* moths and their parasitoids. (A) Normally developing fruits produced by *Epicephala* sp. F (upward arrows) and swollen galls produced by *E*. sp. D (downward arrows). (B) Oviposition marks left by *E*. sp. D on immature swollen galls. (C) Longitudinal section of a fully developed swollen gall with an irregularly developed ovule containing a larva of *E*. sp. D (arrow). Note the airspace around the infested ovule. (D) A braconid wasp ovipositing in a swollen gall. (E) An eulophid wasp resting on a tough gall produced by *E*. sp. C. (F) Relationship between the number of galled ovules per gall and overall gall size (n = 348). White and black circles indicate galls with and without parasitised moth larvae. (G) Logistic regression of the proportions of parasitised moth larvae on galled ovule numbers per gall (n = 380). The area of each circle is proportional to the sample size.

Figure S1. Exit holes made on the fruits of *Phyllanthus reticulatus* by mature *Epicephala* larvae (A) and adult *Bracon* wasps (B). The former is characterised by the jagged edge and a ring of residual fruit epidermal layer through which the moth larvae had penetrated. The latter has smooth edge and lacks any epidermal remains.

Figure S2. Phylogeny of 174 *Epicephala* moths from the *Phyllanthus reticulatus* species complex based on COI gene sequences. The numbers above the branches are

the maximum-likelihood bootstrap values followed by the Bayesian posterior probabilities. Individual moths are labelled with locality names followed by unique numbers. Individuals for which we observed field behaviour are indicated in bold. Mutualistic and parasitic species are coloured green and blue, respectively. The taxonomy of the host with which each species was associated is shown.

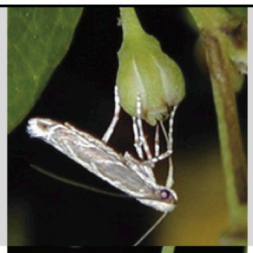
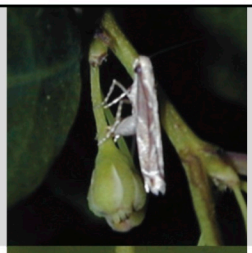
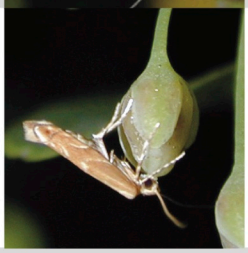
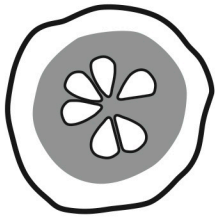
Figure S3. Phylogeny of 49 *Bracon* wasps (right) based on COI gene sequences and their associations with *Epicephala* moth hosts. The wasps were divided into eight distinct clades, which we consider as species. The numbers above the branches are the maximum-likelihood bootstrap values followed by the Bayesian posterior probabilities (given only for nodes above species level). Hatched lines connect each wasp species with its host *Epicephala* species. The two *Bracon* species that attack *Epicephala* associated with plants of the *Phyllanthus reticulatus* species complex (sp. 3 and sp. 8) can be distinguished based on the markings on the dorsal surface of thorax and abdomen in the males: the former possesses dark markings at the centre of first to sixth metasomal terga (T1–T6), both sides of the propodeum and areas around the wing base, whereas the latter possesses only light markings on T3–T6 that are intermitted centrally by non-pigmented area and no mesosomal markings as in sp. 3 (photos). Although *Bracon* wasps are prevalent among *Epicephala* moths, they are absent from *Epicephala* that colonised New Caledonia and those associated with weed *Phyllanthus* (indicated by hatched boxes). Islands and weedy host may have provided *Epicephala* moths with enemy-free space.

Table 1. Intensity of parasitism by braconid and eulophid wasps on *Epicephala* sp. A (pollinator) and *E.* sp. C (gall maker). Data are based on fruit/gall samples collected from six *Phyllanthus reticulatus* individuals at the Hengchun population.

	Fruit (*E.* sp. A)	Gall (*E.* sp. C)	Significance[a]
Selectivity of wasp oviposition			
Natural abundance	282	258	
Braconid ovipositions	27	1[b]	$P < 0.001$
Eulophid ovipositions	1	25	$P < 0.001$
Parasitism rate			
Number examined	176	189	
Fruit/gall with braconid	31	2	$P < 0.001$
Fruit/gall with eulophid	1	95	$P < 0.001$

[a]Significance based on Fisher's exact test.

[b]A single wasp alighted on the gall and attempted oviposition but failed to pierce ovipositors through gall wall.

Moth species	Pollination behaviour	Pollen on proboscis	Oviposition site	Flower fate	No. eggs/ flower	Host species
E. sp. A		+			1	*P. reticulatus* *P. ret.* x *mic.*
E. sp. B		+			3	*P. reticulatus* *P. ret.* x *mic.*
E. sp. C	—	—			1	*P. reticulatus* *P. ret.* x *mic.*
E. sp. D		+			3	*P. microcarpus* *P. ret.* x *mic.*
E. sp. E		±			1	*P. microcarpus* *P. ret.* x *mic.*
E. sp. F		+			1	*P. microcarpus* *P. ret.* x *mic.*